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14. ABSTRACT
We proposed to test PHSCN and PhScN derivatives with modifications of the N– or C–termini, and/or cysteine side chain to determine the initial spatial constraints of the binding pocket of the ligand to its target site. This will aid in the selection of crosslinking agents, modifications, tag additions and strategies for determination of the target site(s) to be performed in Aim 2, and will lay the foundation for determining the structure-activity relationships (SARS) of these inhibitors with the target.

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INTRODUCTION: It has been suggested that PHSCN inhibits metastatic invasion by forming a covalent, disulfide bond with a cysteine residue on the beta1 (β1) subunit of alpha5 beta1 (α5β1) integrin[1]. However, these studies were performed with purified α5β1 integrin, which also produces evidence of covalent bond formation with the α5 subunit (tandem mass spectroscopy data not shown). Hence, the specificity of the reported interaction between PHSCN and the β1 subunit[1] is suspect. Moreover, the cysteine–rich β1 subunit can heterodimerize with 12 distinct alpha integrin subunits[2], forming integrins that function in many pathways. In contrast, the α5 subunit interacts uniquely with the β1 subunit[2] to induce invasion and support adhesion[3-5]; hence it is a much more desirable target. Our funded research is designed to test definitively the hypothesis that both PHSCN and PhScN peptides inhibit α5β1 integrin fibronectin receptor–mediated invasion through noncovalent interaction with α5β1 receptors of SUM149PT and MDA-MB-231 metastatic human breast cancer cells, and to lay the foundation for detailed structure–activity relationship (SAR) studies for both peptides as inhibitors of α5β1–mediated metastatic breast cancer invasion by precisely defining their target site(s) on α5β1 integrin, and by developing an assay that can be used to evaluate *in vitro* degradation rates in plasma, and to monitor *in vivo* plasma levels.

Objective/Hypothesis: Our preliminary data suggested that the invasion inhibitory potencies of the S–acetylated and the S–methylated PHSCN peptide derivatives are increased by over 1,000–fold, relative to PHSCN. In addition, the PhScN peptide is still more potent: preliminary results suggest that it is 10^4 to 10^5–fold more potent than PHSCN at blocking α5β1–mediated invasion. Thus, we hypothesized that the actual invasion–inhibitory interaction of PHSCN with α5β1 integrin involves a noncovalent interaction with the target site, which is promoted by including D–isomers of histidine and cysteine in the PhScN peptide. Hence, covalent, disulfide bond formation with α5β1 integrin is actually a side reaction that *decreases* potency. Because mammalian proteins do not contain D–amino acids, endoproteinses evolved to cleave between L–amino acids only. Since PhScN does not contain 2 linked L–amino acids, it cannot be degraded by endoproteases, usually present at high levels in tumors and increasing with malignancy[6-8]. Thus, in addition to promoting noncovalent interaction with its α5β1 integrin target, the inclusion of D–amino acids at alternate positions is expected to result in increased PhScN potency as a result of preventing endoproteolytic degradation. Our research is designed to achieve the following specific aims.

Specific Aims: Specific Aim 1: To lay the foundation for determining their structure-activity relationships (SARs) as inhibitors of α5β1–mediated invasion[3-5,9,10], we propose to assay PHSCN and PhScN peptide derivatives, having modifications of N– or C–termini, and/or cysteine side chain. Peptides will be tested in quantitative serum–free invasion assays with naturally occurring basement membranes[3-5,9-15].

Specific Aim 2: To identify the binding site for the PHSCN sequence on α5β1 integrin, which will aid in the development of potent PHSCN/PhScN analogs, biotinylated, S-BPM–derivatized PHSCN and PhScN peptides will be photocrosslinked to SUM149PT and MDA-MB-231 cells. Tagged cells and extracts will be screened by fluorescent antibody staining and Western blotting for biotin and α5β1 integrin. The identity of the BPM–modified subunit and sites(s) of modification will then be determined using tandem mass spectrometry to localize the PHSCN/PhScN binding site(s) on α5β1 integrin.

Specific Aim 3: To develop a mass spectrometry–based quantitative assay for PHSCN sequence analogs, including PhScN, and use it to evaluate and enhance their stabilities in human and mouse plasma. This assay is crucial for future in vivo studies where bioavailability and clearance rates are to be monitored.

BODY:

Specific Aim 1

We proposed to test PHSCN and PhScN derivatives with modifications of the N– or C–termini, and/or cysteine side chain to determine the initial spatial constraints of the binding pocket of the ligand to its target site. This will aid in the selection of crosslinking agents, modifications, tag additions and strategies for determination of the target site(s) to be performed in Aim 2, and will lay the foundation for determining the structure-activity relationships (SARS) of these inhibitors with the target.

Selection of Peptides

Table 1 is the list of peptides and their IC50's determined using our *in vitro* invasion assay. Our initial focus was on the modification of the cysteine side chain and the C–terminus. As shown below, S–acetylation or S–methylation of the cysteine side chain in the PHSCN peptide increased its invasion–inhibitory potency by 10,000– to 100,000–fold. Substitution of D–cysteine to form the PhScN peptide, also increased invasion–inhibitory potency by over 100,000–fold, suggesting that PHSCN inhibits invasion by a noncovalent interaction with its target. The only commercially available covalent modification to D–cysteine is S–acetamidomethyl (S-acm). Hence, the invasion–inhbibitory potencies of S-acm–D and –L cysteine in PHSCNGGK and PhScNGGK were compared. As shown below, **covalent modification of the L–cysteine side chain resulted in greatly increased invasion–inhibitory potency; whereas covalent derivatization of the D–cysteine did not further increase its potency**. However, it was determined that modification of cysteine drastically interferes with Kd binding. The S-acm peptide Kd binding values were increased by ~100 fold, with the D-cysteine being more affected than the L-cysteine. This suggests that binding is less efficient with a modified cysteine side chain.

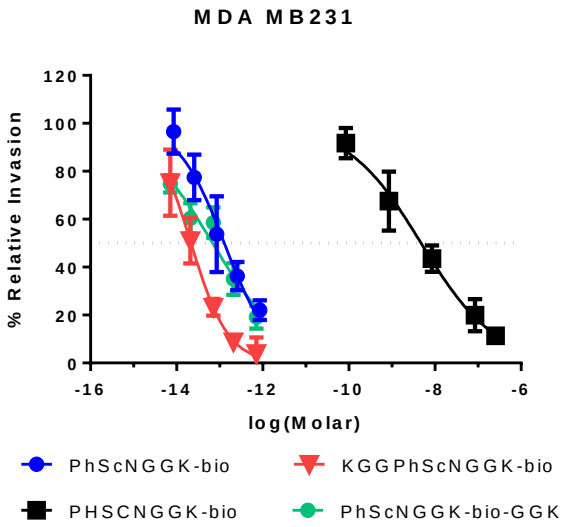
Table 1. Invasion–inhibitory potency of PHSCN and PhScN derivatives. Values expressed in Molarity (M) to adjust for molecular weight of modifications. Peptides highlighted in red were performed in year 2. **C**, commercially synthesized; **D,** derivatization in house of the appropriate commercial parent compound; **TBD**, to be determined; **bio**, biotin tag; **MAP**, multiantigenic peptide; **Me**, S–methylated; **OAc**, S–acetylated; **acm**, S–acetamidomethyl; **BPM,** benzophenone-4-maleimide.

Table 1	C or D	mw	IC_{50} M	Kd µM	L or D isomer	Modification Region
Ac-PHSCN-NH_2	C	598	2.8 E-8		L	parent
Ac-PHSCNGGK-bio	C	1180	4.8 E-9	0.03	L	tail
Ac-PHSC(Me)N-NH_2	C	612	5.3 E-13		L	side
Ac-PHSC(OAc)N-NH_2	C	640	1.4 E-14		L	side
Ac-PHSC(acm)NGGK-bio	**C**	**1537**	**2.3 E-14**	**5.6**	**L**	**Tail + side**
Ac- PHSC(BPM)GGK-bio	D	1436	TBD		L	Tail + side
Ac-PHSCNGGK-MAP	C	7575	2.0 E-11		L	Tail (MAP)
Ac-PhScN-NH_2	C	598	2.2 E-14		D	parent
Ac-PhScNGGK-bio	C	1180	1.3 E-13	0.03	D	tail
Ac-PhSc(BPM)GGK-bio	D	1436	1.2 E-14		D	Tail + side
Ac-PhSc(acm)N-GGK-bio	**C**	**1537**	**0.7 E-14**	**17**	**D**	**Tail + side**
Ac-PhScNGGK-MAP	C	7575	1.7 E-20		D	Tail (MAP)
Ac-PhScNGGK-bio-GGK	**C**	**1480**	**6.9 E-14**	**0.02**	**D**	**Tail + ext**
Ac-KGGPhScNGGK-bio	**C**	**1480**	**2.1 E-14**	**0.02**	**D**	**Head + Tail**

C- and N-Terminus Extended Peptides

The addition of the biotin and the creation of a MAP (multi-antigenic peptide) on the C-terminus and the retention of activity suggest that the "tail" of the peptide has sufficient room for modification. The low Kd value of 0.03 also suggests that the addition of the biotin group to the parent compound has little effect on binding. Two new peptides Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio were commercially synthesized. These peptides have three additional amino acid residues at either the N– or C–terminus to introduce an amino group to allow for a new crosslinking site. The introduction of these new residues did not reduce the invasion– inhibitory potency, and furthermore did not affect Kd binding (Fig 1 and Table 1). These new peptides will allow for an array of crosslinking reagents to be evaluated in Aim 2.

Figure 1. Invasion inhibition with C- and N-terminus modified peptide ligands.



Activity of C- and N-Terminus Extended Peptides Derivatized with Crosslinking Agents

The new peptides (Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio) were self–derivatized with the amine modifying portion of heterobifunctonial crosslinkers, and tested in the invasion assay at a single dose. Comparison to the underivatized peptide suggests that the addition of the crosslinker to either the N– or C–terminus does not affect invasion potency (Table 2).

Table 2. Invasion inhibition with C– and N–terminus extension to include an available amine group. Parent peptide was modified with the appropriate crosslinker and converted to molarity to adjust for changes in mass. A single point value was collected and compared to the underivatized peptide at a similar concentration.

	Å arm	**Total mw**	**pM**	**Ac-PhScNGGK-bio-GGK**	**Ac-KGGPhScnGGK-bio**
No crosslinker	0	1480	0.7	21%	18%
Sulfo-LC-SDA	12.5	1920	~1.6	17%	17%
Sulfo-SANPH	18.2	1972	~1.5	19%	14%
ANB-NOS	7.7	1785	~1.7	15%	6%

Structure-Activity Relationships (SARS) experiments

Effects of D–amino substitution of cysteine and/or histidine in Ac-PHSCN-NH_2 were also tested (Fig 2). Substitution of either cysteine or histidine imparted a 100– or 1000–fold increase in potency, respectively. The substitution of both cysteine and histidine produced the most potent version of the peptide, with over 100,000–fold increase in potency. Moreover, it appears that the orientation of the cysteine side chain and histidine ring on the same side of the peptide backbone is key. In addition, each residue in Ac-PhScN-NH2 was systemically replaced with alanine and tested in the invasion assay (Table 3). Substitution of any amino acid with alanine eliminated the potency of the peptide, and further suggests that the sequence of the compound is just as important. In addition the D–amino substitution of both the cysteine and histidine produces a sequence of alternating D and L amino acids, making the peptide endoproteinase–resistant, and increasing its stability.

Figure 2. Invasion inhibition by peptides with D–amino acid substituted cysteine and/or histidine.

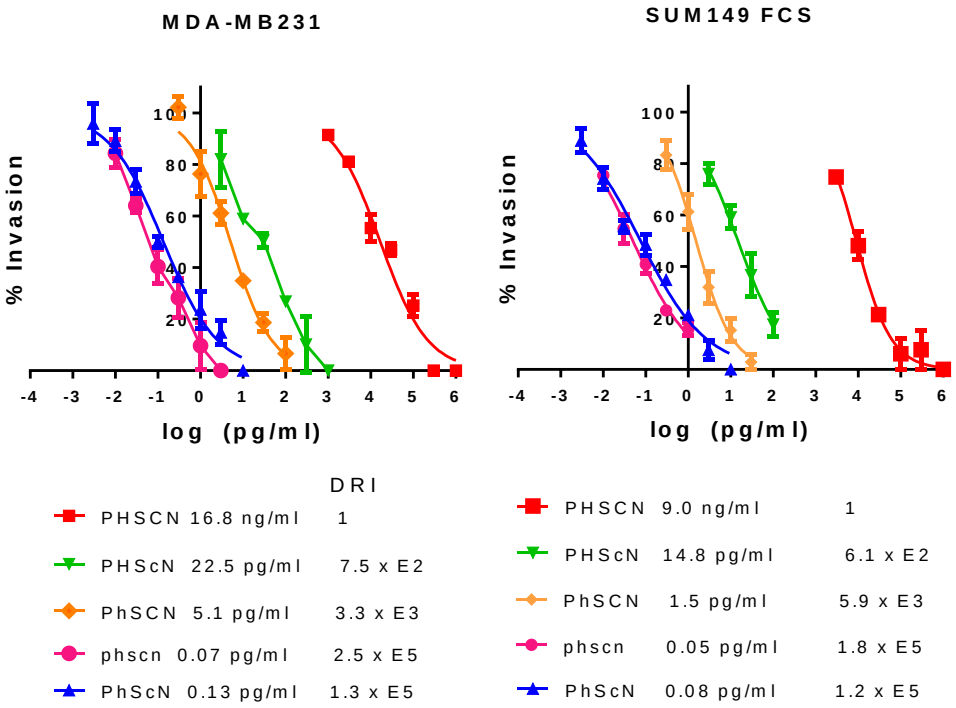


Table 3. Effects of Alanine substitution on invasion inhibition for each position in Ac-PhScN-NH_2

	Alanine Scan	
	MDA MB211	**Sum149PT**
1 pg/ml	**Rel. % Inv**	**Rel. % Inv**
PhScN	0%	0%
PhScA	94%	99%
PhSAN	89%	123%
PhAcN	100%	118%
PAScN	95%	112%
AhScN	99%	94%

The invasion–inhibitory potencies of PhScN peptides with selective modifications were also tested (Table 4). These modifications also prevented invasion inhibition by PhScN, supporting the importance of the peptide's sequence and spatial constraints of the head and cysteine side chain as shown below, and in Fig 2 and Table 3.

Table 4 Invasion–inhibitory potencies of select modifications of Ac-PhScN-NH_2, including N–butyl (Bu–), D–asn (PhScn), and D–met (PhSmN), as well as the scrambled sequence control, Ac-NcPhS-NH_2.

Compound 1 pg/ml	% Rel. Inv	Rationale for Modification
Ac-PhScN-NH2	0%	parent
Bu-PhScN-NH2	83%	Head space
Ac-PhScn-NH2	102%	D-amino substitution of Asn
Ac-PhSmN-NH2	97%	D-Cysteine side chain by methionine
Ac-NcPhS-NH2	109%	Scrambled D-amino control

Specific Aim 2
Identification of the binding site for the PHSCN/PhScN sequence on α5β1 integrin using protein crosslinking techniques and subsequent identification of the protein and peptide location by tandem mass spectrometry.

Crosslinking with benzophenone-4-maleimide(BPM)–derivatized peptides. Analysis by Western Blotting.
PHSCN binds specifically to activated α5β1 integrin. Determination of the subunit and the exact binding site location is the goal of this proposal. There are multiple activation states of integrins, which are achieved by a combination of divalent cations and/or specific ligands. Our first attempt in year 1 at crosslinking the peptide to its target protein was to replicate the conditions used for *in vitro* invasion assays. Serum–starved cells were stimulated by serum, and were then allowed to incubate with the BPM–derivatized peptides, followed by UV crosslinking to activate the BPM tag. To simplify and avoid losses, no washing steps were performed. As shown in Figure 3, the primary biotin–labeled band is much smaller than the subunits of α5β1 integrin and their expected mass, assuming full glycosylation of the asparagine in the primary sequence (Table 5).

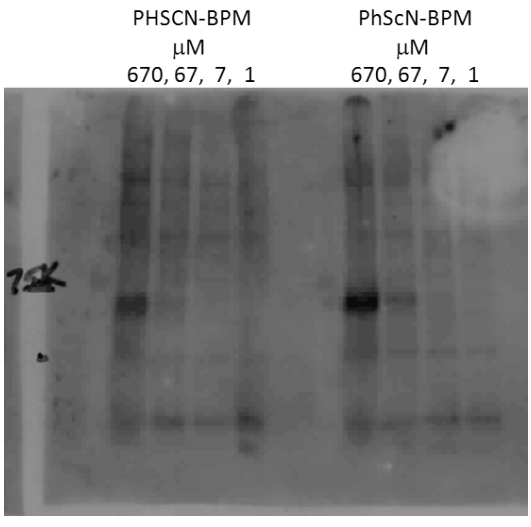


Figure 3. SUM149PT cells, serum starved overnight. Cells suspended into medium at 37 C for 45 minutes. 40,000 cells were incubated with various concentrations of BPM–derivatized peptides for another 20 min. UV irradiation for 45 minutes at room temp. 7.5% SDS-PAGE, blotted with Anti-biotin MAb.

Table 5

Table 2	Da Primary sequence	Da fully glycosylated
α5 subunit	114,536	150,964
β1 subunit	88,465	119,689

The presence of the major low molecular weight biotin labeled protein suggests that two possible scenarios may have occurred:

either the BPM–derivatized PHSCN or PhScN peptide has formed an intermolecular crosslink with a nearby protein instead of an intramolecular crosslink within the target protein, or the presence of 10% serum introduced a nonspecific binding target. Considering the high concentration of the peptide necessary to observe this low mass complex, these data suggest: 1) nonspecific binding, and 2) that BPM–derivatized peptides have very high dissociation constants, (K_d's). To reduce or eliminate nonspecific binding, further attempts at crosslinking were performed after integrin activation by divalent cations. To ascertain whether the modified peptides had altered binding properties because of steric hindrances not detected by the functional *in vitro* invasion assay, the binding constants (K_d) of the relevant compounds were completed in year 2 (Table 1). These assays showed that BPM–derivatized PhScN and PHSCN peptides had greatly elevated Kd values, suggesting steric hindrance.

Crosslinking after activation with divalent cations

The crosslinking experiment, whose results are shown in Figure 3, was repeated using divalent cations to activate integrin and a varying amount of cells to increase signal. As shown in Figure 4 below, the use of divalent cations to activate α5β1 eliminated the prevalent biotin–labeled low molecular weight complex, suggesting that this was indeed due to nonspecific binding to the serum proteins at the high concentrations of peptide required. The presence of bands in the region of interest is promising; however the amount observed suggested that the Kd binding constants of the BPM peptide derivatives were much greater than the underivatized parent. The presence of bands only in the L lanes (L-cysteine) (Fig 4) further suggested that there are more steric hindrances in binding for the D lanes (D-cysteine). These observations were confirmed with the completed binding assays.

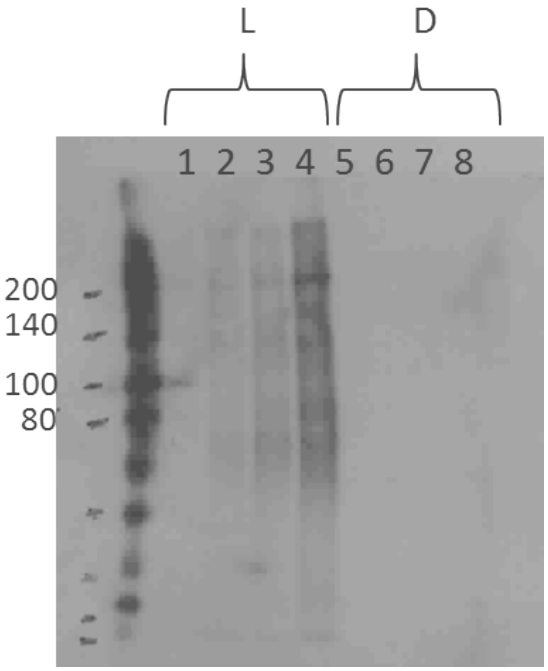


Figure 4. Western blot with Anti-biotin of a 7.5% SDS-PAGE of varying amounts Mn^{2+} activated MDA-MB-231 cells incubated with 50 μM of BPM derivatized peptide. Ac-PHSC(BPM)NGGK-bio(L) and Ac-PhScN(BPM)NGGK-bio(D) denote BPM ligand. The cell quantity varied for each lane ln 1,5 50K; ln 2,6 100K; ln 3,7, 200K and ln 4,8, 500K.

As proposed in the prior progress report, cross-linking with the BPM peptides was continued while the new C– and N–modified peptides were being synthesized and characterized. Attempts to scale the BPM experiments for protein enrichment and identification by MS/MS were unsuccessful, and hence abandoned. Furthermore, as previously stated, the binding constants (Table 1) confirmed that modification of the cysteine side chain did perturb the binding constant, more so for D-cysteine than L-cysteine. These observations supported the decision to abandon this approach.

The new, extended C– and N–terminus–derivatized Ac-PhScNGGK-bio-GGK (bio-GGK)– and Ac-KGGPhScNGGK-bio (KGG-bio)– peptides have been characterized and were found to retain full activity and binding properties (Table 1). The lysine side chain in the extension was first derivatized with the amine reactive region of the heterobifunctional crosslinkers. Samples were tested for invasion–inhibitory potency (Table 4), and were found to retain activity after the first derivatization. Cells were incubated with the crosslinked peptides and then exposed to UV light to activate the nonspecific region of the crosslinkers. Different length crosslinkers were tested with both versions of the extended peptides. The results are shown in Fig. 5. The initial assessment is that the primary band is the same for all the crosslinkers used, and that either

the bio–GGK version or the KGG–bio version of the peptide can be employed. *Note:* Lanes 1 and 2 are very faint but denote the same bands as seen in lanes 3–6. (not shown). Work is under way to improve the derivatization of the peptides, cleanup, and optimization of the photoactivation of the crosslinkers.

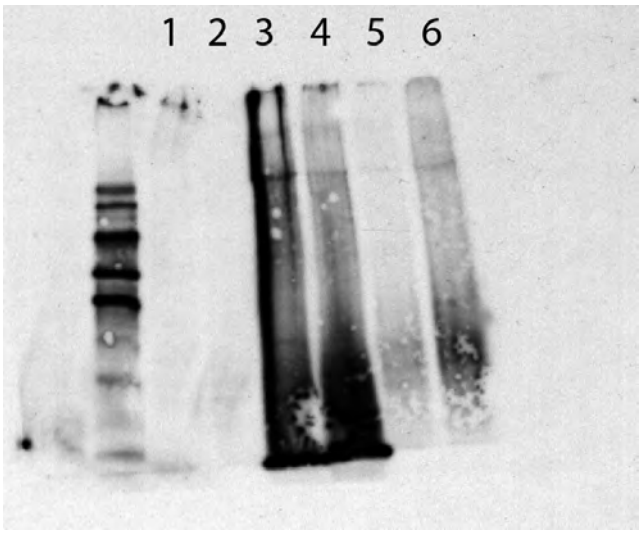


Fig 5. Photoactivatible crosslinkers with the C- and N-terminus peptides. Western blot by detection with anti-biotin HRP. Lane 0, biotinylated mw markers.

Lane	Peptide	Crosslinker	Length
1	bio-GGK	sulfo-LC-SDA	12.5
2	KGG-bio	sulfo-LC-SDA	12.5
3	bio-GGK	sulfo SANPAH	18.2
4	KGG-bio	sulfo SANPAH	18.2
5	bio-GGK	ANB-NOS	7.7
6	KGG-bio	ANB-NOS	7.7

Our collaborator, Dr. Philip Andrews has developed a cell–permeable cross–linker, DC4, which spans 15 angstroms and targets amine groups. DC4 was found to have a minor reaction with histidine and cysteine residues on the shortened parent version of the peptide in year 1. After development of the new extended peptides, DC4 was retried in year 2 as designed. As shown to the right in Figure 6, the primary band appears to be in the same region of interest, as seen with the photoactivatable compounds in Fig 5. For the Western blot shown in Fig. 6, the proteins were extracted with 100 mM octyl-β-D–glycopyranoside (OBG) in RIPA buffer and the supernatent enriched by streptavidin agarose. As shown here, the majority of the labeled material remained in the pellet. Since DC4 will react with any free amine, over–crosslinking can occur, trapping proteins in complexes. The presence of biotinylated sample remaining in the pellet indicates that more aggressive solubilization is necessary to extract the sample prior to enrichment; however that must be balanced with compatibility of binding conditions to streptavidin. The alternative is to digest the proteins and then enrich the peptides. Both methods will be employed in subsequent studies.

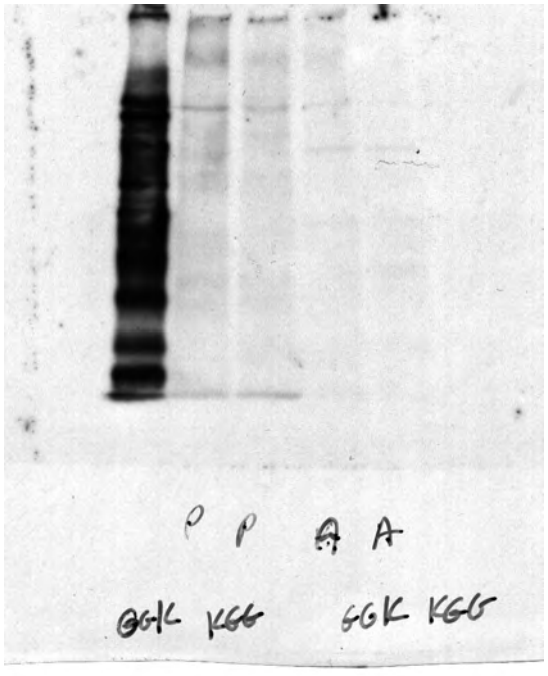


Figure 6. Western blot demonstrating crosslinking with DC4 and both the bio–GGK and KGG-bio peptides. The P denotes the pellet sample and the A denotes enrichment of the sample with biotin tag detected by anti-biotin HRP. Lane 1, biotinylated mw markers.

Summary of crosslinking experiments

Our first approach was to use the cysteine side chain modified BPM–derivatized peptides. As seen in Fig 3 and 4, and confirmed by binding assays in year 2 (Table 1), efficient crosslinking via the cysteine was not achieved. It was also necessary to evaluate and determine the conditions necessary to promote integrin activation and efficient binding. In year 2 the extended peptides were characterized and found to be more suitable for crosslinking. As shown in Figs 5 and 6, a dominant labeled protein is present on the anti-biotin HRP probed Western blot, and attempts at scaling, enrichment and identification are in progress.

Results:

1. Cysteine side chain derivatized peptides retained favorable activity but the binding properties were unfavorably changed. The characterization of two new peptides Ac-PhScNGGK-bio-GGK and Ac-KGGPhScNGGK-bio allows for additional crosslinkers to be evaluated. These peptides were found to not introduce any binding specificity issues, and the lower Kd shows more efficient binding. The additional amine group on the new lysine also allows for a considerable array of both homobifunctional amine reagents as well as a selection of heterobifunctional photoactivatable crossslinkers.

2. Activation of integrin by serum introduces additional proteins that can nonspecifically bind to the peptide compound at the high concentrations required to initiate high levels of binding to the active site. Use of divalent cations eliminates the nonspecific binding problem; however the steady state amount of activated integrin is much lower than the amount obtained from serum starvation and subsequent stimulation. The ligand PHSRN consists of the specific sequence in fibronectin that stimulates activation of the $\alpha 5\beta 1$ integrin. Quantities of this peptide have been obtained and will be used to stimulate activation of integrin after serum starvation.

3. The effect of the increased levels of integrin activation from stimulation after serum starvation is relatively short. The maximal effect occurs 1 hr after stimulation with either serum or PHSRN, and is completely gone by 4 hrs. Serum starvation and stimulation by the ligand PHSRN will be used in combination with divalent cations to promote upregulation of activated $\alpha 5\beta 1$ integrin.

4. Detection of crosslinking has been seen only on Western blots that have been over–developed. Sample enrichment from BPM–peptide experiment using 10^7 cells have not yielded enough material to analyze. Published papers show that only the activated form of $\alpha 5\beta 1$ integrin is receptive to the compound. Attempts to increase the amount of activated integrin are being used, but have their limitations. The remaining option is to continue increasing the amount of cells used and continue enrichment strategies.

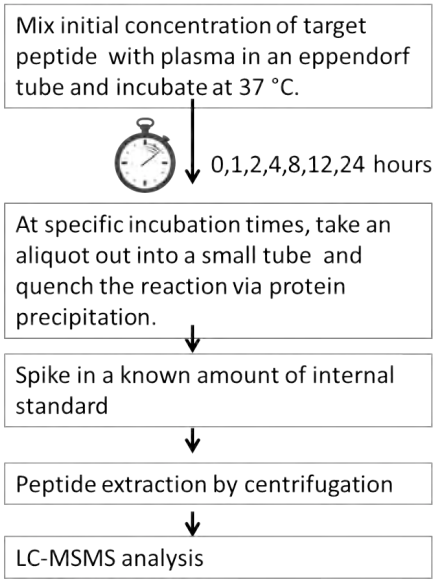
5. Assessment of the DC4 and photoactivatable crosslinkers on Western blots show that the extended bio–GGK and KGG–bio peptides with any of the four crosslinkers appear to all generate the same crosslinked complex. As seen with the MAP–peptide, the tail region has the least spatial constraint; hence the bio-GGK–derivatized peptide is the better choice for continued work, effectively reducing the number of combinations by half. Our colleague has had extensive experience with DC4, however the various photoactivatable crosslinkers will each bring their own mass spectrometry challenges.

6. Work is in progress with scaling to 10^8 cells with the DC4 crosslinker and the photoactivatable crosslinkers with the bio–GGK peptide. Enrichment with magnetic beads will be used. It is hopeful that sufficient material will be obtained to allow for masss spectrometry analysis and definition of the target.

Specific Aim 3
Develop a mass spectrometry–based quantitative assay for PHSCN analogs, including PhScN, and evaluate their stabilities in human and mouse plasma.

Experimental design for quantitative assay of PHSCN analogs
An experimental approach based on mass spectrometric analysis to evaluate the stabilities of PHSCN analogs in human and mouse plasma has been designed, as outlined below in Scheme 1. A known amount of target peptide is mixed with plasma and incubated at 37 °C for up to 24 hours. At specific incubation time points an aliquot is transferred to a small tube and protease reactions are quenched by adding three volumes of 50% acetonitrile with 0.5%TFA. Precipitated plasma proteins are removed by centrifugations, and a known amount of an internal standard is spiked into the supernatant containing the peptides, in order to increase the accuracy of quantitative measurements of LC-MS by reducing variances associated with sample preparations. MRFA (Met-Arg-Phe-Ala, monoisotopic molecular mass = 523.247.), a stable synthetic peptide with similar molecular mass to the target peptide, Ac-PHSCN-amide (monoisotopic molecular mass = 597.2328), is added into samples prior to LC-MS analysis. Stabilities of target peptides in plasma will then be evaluated by LC-MS.

Mass spectrometry procedure
The LTQ-Orbitrap XL(Thermo Finnegan) coupled to a nanoLC(Exigent) and an autosampler in the Andrews laboratory provides high mass resolution and accuracy for analyses separated on a capillary column at a flow rate of 200 nL/min, which assures confident identification of target molecules. Extracted peptides from plasma are reconstituted in 0.1% TFA, loaded into a C18 trap by autosampler, and washed for 6 min at 5 microL/min with 0.1% TFA in water delivered by the LC loading pumps. Trapped peptides are introduced by switching valve into a capillary column (75 micro-m x 15 cm) custom-packed with C18 resin (3 micron), and separated over 60 min gradient at a flow rate of 200 nL/min. Analytes are introduced into the LTQ-Orbitrap via an electrospray device (Triversa Nanomate, Advion). MS and MSMS data are acquired in data–dependent mode for ions within 400-800 m/z.



Scheme 1. Experimental workflow

Figure 7 (next page) shows the total ion chromatogram (TIC) of LC-MSMS analysis of PHSCN and the internal standard MRFA.

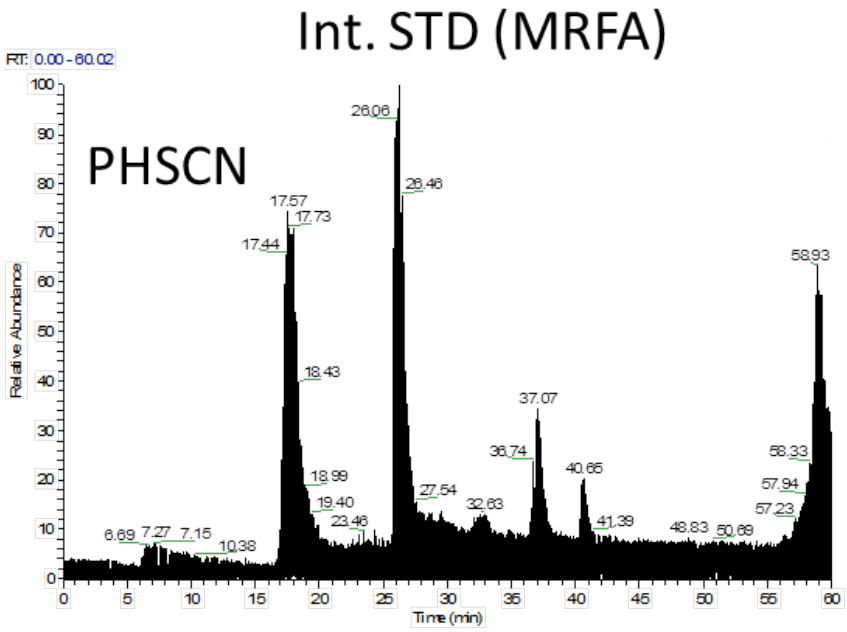


Figure 7. TIC from an LC-MSMS of PHSCN and MRFA mixture.

Quantitative assay of target peptides

Peptide quantification can be achieved by ion intensities in MS, or by MSMS spectral counts in data–dependent acquisition mode. These two approaches are well established, label–free quantification methods for peptides and proteins by LC-MSMS, which can be readily applied to a bioanalytical assay of therapeutic peptides. We are currently testing a new assay method, an MRM-like analysis via data–independent acquisition mode in the LTQ-Orbirap, in which ion packets within a mass range of 5 or 10 Da, accumulated for a set time, are co–fragmented. One or multiple fragment ions specific to the target peptide can be monitored in MSMS based on highly accurate precursor ion mass and LC retention time. This MRM-like approach can provide a highly selective and sensitive bioanalytical assay under conditions of high chromatographic interference.

Study of collision–induced dissociation (CID) fragmentation of Ac-PHSCN-NH_s in online LC-LTQ-Orbitrap

Ac-PHSCN-NH_2 introduced into the mass spectrometer via a nanoESI device (Triversa Nanomate) was predominantly observed as singly protonated form at 598.2328 m/z. However, in online LC-MS, the peptide was observed in doubly charged dimeric forms at 597.23 m/z and 589.23 m/z. The peptide dimer ions were characterized by CID that revealed disulfide-bond formation between two peptides that was catalyzed on C18 LC, as shown in a CID spectrum of 597.23 m/z. These results are shown in Figure 8, on the next page. This is a potential source of interference with the assay that can be easily addressed by incorporating reduction and alkylation of the Cys residues into the protocol prior to LCMSMS.

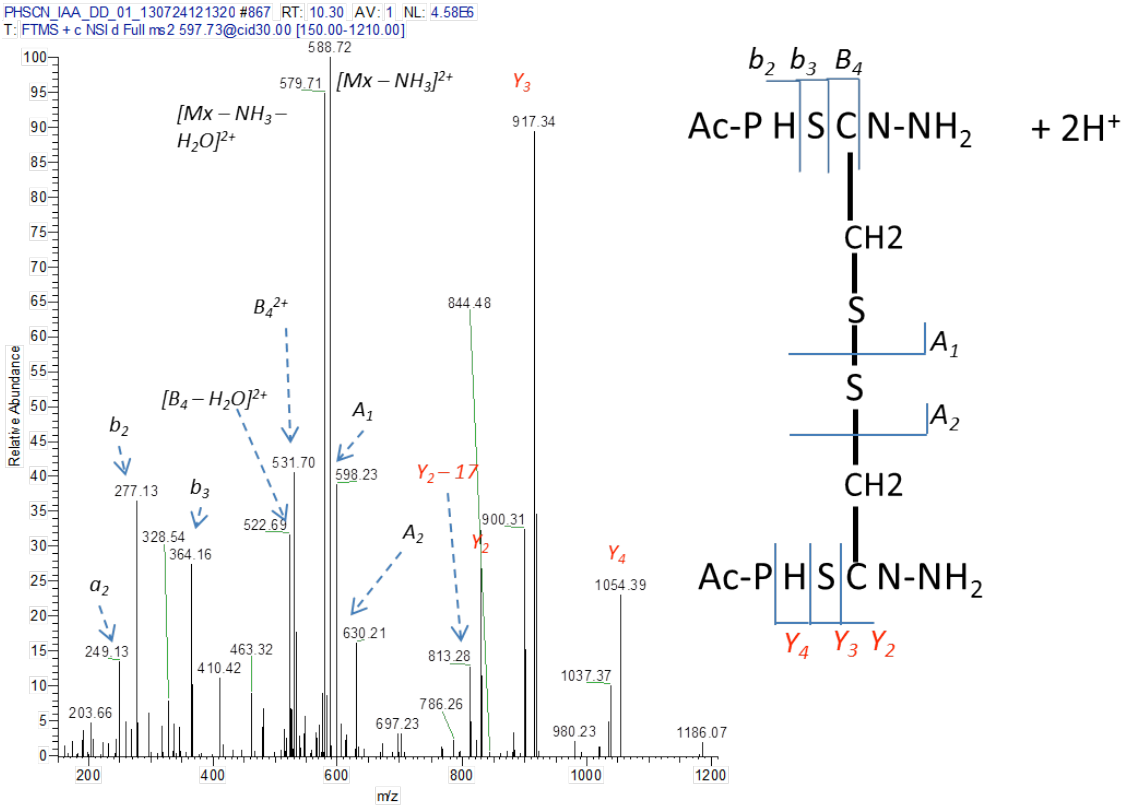


Figure 8. CID fragmentation of doubly–charged ion at 597.73 m/z (the second isotope to 597.23 m/z). Fragment ions B4, A2, Y2,Y3, and Y4 containing S-S cross-linked peptides confirm disulfide bond formation between two thiols of cysteine.

KEY RESEARCH ACCOMPLISHMENTS:

1. Determination of invasion–inhibitory potencies for a total of 14 PHSCN and PhScN derivatives, targeting $\alpha 5\beta 1$ integrin–mediated invasion of naturally serum–free basement membranes by 2 metastatic human breast cancer cell lines, SUM149PT and MDA-MB-231. Demonstration that covalent S–modification by several different moieties increases invasion–inhibitory potency by 100,000– to 1,000,000–fold, and that a 1,000,000–fold increase in potency is also obtained by substitution of D–His and D–Cys. **These results demonstrate that the reason for the greatly increased invasion–inhibitory potency of PhScN is the suppression of covalent disulfide bond formation between the PHSCN sequence and its integrin target, and hence they are a key step in the determination of the PhScN target site on $\alpha 5\beta 1$ integrin receptors of breast cancer cells.**
2. Determination of invasion–inhibitory potencies of PHSCN and PhScN peptides, containing BPM–derivatized cysteine or D–cysteine. The greatly increased invasion–inhibitory potencies of the BPM–modified PHSCN and PhScN peptides also confirm the noncovalent nature of invasion inhibition by the PhScN peptide.
3. The importance of the D-amino substitution in Ac-PhScN-NH_2 was further explored. The substitution of either cysteine or histidine imparted a 100– or 1000–fold increase in potency, respectively. In combination, the substitution of both cysteine and histidine produceed the most potent version of the peptide, with a 100,000–fold increase in potency. Substitution of any amino acid with alanine eliminated the potency of the PhScN compound, further suggesting that each amino acid is key to PhScN potency.
   Research accomplishments 1, 2 and 3 complete Specific Aim 1 of the SOW.

4. Determination of binding constants for PHSCNGGK-Bio and PhScNGGK-Bio on MDA-MB-231 and SUM149PT cells, and demonstration of their similarity provided the basis for acquiring information on any steric hindrances introduced by modification of the ligands not detected in functional invasion assays. Furthermore, it was found that PHSCNGGK-Bio and PhScNGGK-Bio compete for the same binding site on SUM149PT and MDA-MB-231 cells. The results of these binding assays, in conjunction with the functional invasion inhibition results shown in Table 1, allowed a more complete profile of the peptide ligand properties and their functional effects on $\alpha5\beta1$ integrin–mediated invasion. Moreover, these results provided key support of the hypothesis that the increased potency of the PhScN peptide results from suppression of covalent, disulfide bond formation with the integrin target.
5. Crosslinking with BPM–derivatized peptides, after integrin $\alpha5\beta1$ activation with divalent cations, and analysis by Western blotting. Limited success was achieved with the BPM–derivatized peptides. It was found that the modification of the cysteine perturbed the binding constant, and has been since abandoned. New peptides containing additional amino acids on either the C– or N–terminus were characterized and found to not interfere with binding or potency. These new peptides with a lysine group allow use of a large array of hetero-bifunctional crosslinkers in Aim 2. Work is in progress to scale up the crosslinking experiments to acquire sufficient material to identify by mass spectrometry.

Research accomplishments 4, and 5 provide key support for accomplishing Specific Aim 2 of the SOW.

6. Demonstration of CID fragmentation of Ac-PHSCN-NH_2 in online LC-LTQ-Orbitrap indicates that development of a mass spectrometry–based quantitative assay for PHSCN analogs, including PhScN, for the purpose of evaluating their stabilities in human and mouse plasma (Specific Aim 3) is near completion. Our results show that a mass spectrometry-based assay is a viable approach to degradation analysis.

REPORTABLE OUTCOMES: None to date.

CONCLUSION: The PhScN peptide is a highly potent inhibitor of $\alpha5\beta1$ integrin–mediated, serum–induced invasion because it suppresses covalent, disulfide bond formation with its target. Moreover, results of competition assays show that it targets the same region of $\alpha5\beta1$ integrin as the PHSCN peptide parent. A mass spectrometry–based assay is a viable approach to degradation analysis.

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